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RESEARCH PAPER

Stable isotope-labelled morphine to study *in vivo* central and peripheral morphine glucuronidation and brain transport in tolerant mice

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BACKGROUND AND PURPOSE

Chronic administration of medication can significantly affect metabolic enzymes leading to physiological adaptations. Morphine metabolism in the liver has been extensively studied following acute morphine treatment, but such metabolic processes in the CNS are poorly characterized. Long-term morphine treatment is limited by the development of tolerance, resulting in a decrease of its analgesic effect. Whether or not morphine analgesic tolerance affects *in vivo* brain morphine metabolism and blood–brain barrier (BBB) permeability remains a major question. Here, we have attempted to characterize the *in vivo* metabolism and BBB permeability of morphine after long-term treatment, at both central and peripheral levels.

EXPERIMENTAL APPROACH

Male C57BL/6 mice were injected with morphine or saline solution for eight consecutive days in order to induce morphine analgesic tolerance. On the ninth day, both groups received a final injection of morphine (85%) and d3-morphine (morphine bearing three ^{2}H; 15%, w/w). Mice were then killed and blood, urine, brain and liver samples were collected. LC–MS/MS was used to quantify morphine, its metabolite morphine-3-glucuronide (M3G) and their respective d3-labelled forms.

KEY RESULTS

We found no significant differences in morphine CNS uptake and metabolism between control and tolerant mice. Interestingly, d3-morphine metabolism was decreased compared to morphine without any interference with our study.

CONCLUSIONS AND IMPLICATIONS

Our data suggests that tolerance to the analgesic effects of morphine is not linked to increased glucuronidation to M3G or to altered global BBB permeability of morphine.

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Abbreviations

ACN, acetonitrile; BBB, blood–brain barrier; d3-morphine, morphine bearing three ^{2}H; LOD, limit of detection; M3G, morphine-3-glucuronide.; M6G, morphine-6-glucuronide; MPE, maximal possible effect; MRM, multiple reaction monitoring mode; SKIE, secondary kinetic isotope effect; TDM, therapeutic drug monitoring; TLR4, toll-like receptor 4; UDPGA, UDP glucuronic acid; UGT, UDP-glucuronosyl-transferase

Introduction

Chronic administration of medication (Sweeney and Bromilow, 2006) such as anticancer drugs (Hu *et al*., 2015), pain killers [codeine (Antonilli *et al*., 2012)], antibiotics [rifampin (Lee *et al*., 2006)] or antiepileptic drugs [phenobarbital (Sakakibara *et al*., 2016)] can crucially affect metabolic enzymes and ultimately lead to physiological adaptations. Among painkillers, opiate metabolism in the liver has been extensively studied following acute **morphine** treatment (Smith, 2009; Chau *et al*., 2014). Although morphine's analgesic effect mainly involves binding to the **μ opioid receptors** in the CNS, its central metabolism is poorly characterized (Laux-Biehlmann *et al*., 2013). Previous studies have demonstrated how long-term administration results in tolerance, that is, a decrease in the analgesic effect of morphine (Williams *et al*., 2013). While progress has been made towards understanding the cellular basis of morphine analgesic tolerance (Williams *et al*., 2013), whether it affects the *in vivo* blood–brain barrier (BBB) permeability and/or the CNS metabolism of morphine remains a major unanswered question (Yousif *et al*., 2008; Strazza *et al*., 2016; Chaves *et al*., 2017). Intriguingly, naloxone still induces hyperalgesia and precipitates withdrawal symptoms in both tolerant and dependent animals and patients (Morgan and Christie, 2011). This implies that a pool of μ receptors remains functional in such states. Thus, the possibility that chronic morphine treatment leads to tolerance through up-regulation of central morphine catabolism and overproduction of its pro-algesic and pro-inflammatory metabolite, morphine-3-glucuronide (M3G) (Lewis *et al*., 2010; Roeckel *et al*., 2016) represents an interesting hypothesis.

The major route of morphine metabolism is glucuronidation and relies on the **UDP-glucuronosyl-transferase** (UGT) family of enzymes expressed in hepatocytes (Stone *et al*., 2003), neurons and glial cells of the CNS (King *et al*., 1999). In mice, UGT2B36 mainly converts morphine to M3G, whereas morphine-6-glucuronide (M6G) is absent (Oguri *et al*., 1990; Kuo *et al*., 1991; Milne *et al*., 1996; Zelcer *et al*., 2005; Xie *et al*., 2017).

Monitoring morphine metabolism during chronic treatment represents a challenge due to the residual presence of precursors or metabolites from previous administrations (Rubovitch *et al*., 2009). Chronic administration of a drug can also change drug pharmacokinetics. Furthermore, alterations of brain metabolism cannot be distinguished from peripheral metabolism or a change in BBB permeability. Molecules labelled with stable isotopes have been used for decades, in both animals and humans, to study pharmacokinetic differences induced by chronic treatments (Mutlib, 2008; Schellekens *et al*., 2011). For example, in neonates under maintenance therapy, labelled phenobarbital and phenytoin were used to determine clearance, half-life and volume of distribution of the drugs without interference with the ongoing therapy (Malik *et al*., 2003).

The present study used stable isotope-labelled morphine to study BBB permeability and metabolism of morphine after chronic treatment. We have shown that tolerance to the analgesic effects of morphine in mice was not linked to metabolic changes or alterations in the drug's overall BBB permeability.

Methods

Animals

All animal care and experimental procedures were in accordance with European directives (86/609/EEC) and were approved by the regional ethics committee and the French Ministry of Agriculture (licence no. 00456.02 to Y. G.). Studies are reported following the ARRIVE Guidelines for reporting experiments involving animals (Kilkenny *et al*., 2010; McGrath and Lilley, 2015). Experiments were performed with 45-day-old adult male C57BL/6 mice weighing 24 ± 3 g (Charles River, L'Arbresle, France). In each group, equal numbers of male mice were used. Animals were given free access to food and water (autoclaved tap water), with a 12 h light–dark cycle at a temperature of 22°C ± 2°C. Cage bedding was from Anibed (Pontvallain, France; reference AB3) and food from SAFE (Augy, France; reference A04). Mice were kept group-housed at five per cage (Type II cage, 370 cm^2, height 14 cm). Mice were habituated to their experimental environment and handled for 1 week before starting the experiments. Particular efforts were made to minimize the number of mice and the pain they experienced.

Experimental design

Experiments were carried out in a randomized and blinded manner, and statistical analyses were done in a manner blind to treatment. At least three technical replicates were used for *in vitro* experiments. Mice were assigned an identity number and assigned to groups randomly so that the experimenter was blind to treatment when performing behavioural assays. We chose to use 10 animals per group to ensure sufficient statistical power while reducing the number of animals as much as possible. Because morphine analgesia and analgesic tolerance have been shown to differ between males and female animals (Loyd and Murphy, 2014), and to avoid using too many animals by doing all experiments on both sexes, we chose to only use male mice.

Acute morphine and d3-morphine injection

All i.p. injections of morphine and d3-morphine were performed in the morning (light phase at 10 AM). Mice were weighed and then i.p. injected with 10 $mg \cdot kg^{-1}$ morphine

(Euromedex, Souffelweyersheim, France) or pure d3-morphine ((5a, 6a)-7,8-didehydro-4,5-epoxy-17-(methyl-d3) morphinane-3,6-diol; Alsachim, Illkirch Graffenstaden, France) diluted in NaCl 0.9% (w/v). Mice were killed 90 min later (see below).

Tolerance induction and d3-morphine injection

Mice were injected (i.p.) with 10 mg·kg^{-1} of morphine diluted in NaCl 0.9% (w/v), or an equivalent volume of saline for eight consecutive days (Singh *et al.*, 2003). On the ninth day, both groups received a final injection of 10 mg·kg^{-1} of morphine (85%) and d3-morphine (15%, w/w; Figure 1A). As additional controls for tolerance induction, two other groups of mice received only chronic morphine or chronic saline for nine consecutive days. Because our objectives were to determine if morphine glucuronidation in the CNS and BBB permeability were affected, mice were killed 90 min after the last injection (see the section 'Tissues, plasma and urine recovery'). This 90 min time point has been chosen because it represents a good compromise between morphine's half-life in the blood and in the CNS of mice (Webster *et al.*, 1976; Xie *et al.*, 2000; Dalesio *et al.*, 2016).

Nociception assays

Tolerance development was assessed every day 30 min after each injection. Mice were placed on a hot plate (Bioseb, Vitrolles, France) set at 54°C for the measurement of heat nociceptive responses. Latency before the first sign of hind paw discomfort (hind paw licking or jumping) was recorded with a 30 s cut-off. Prior to morphine or saline injections, a

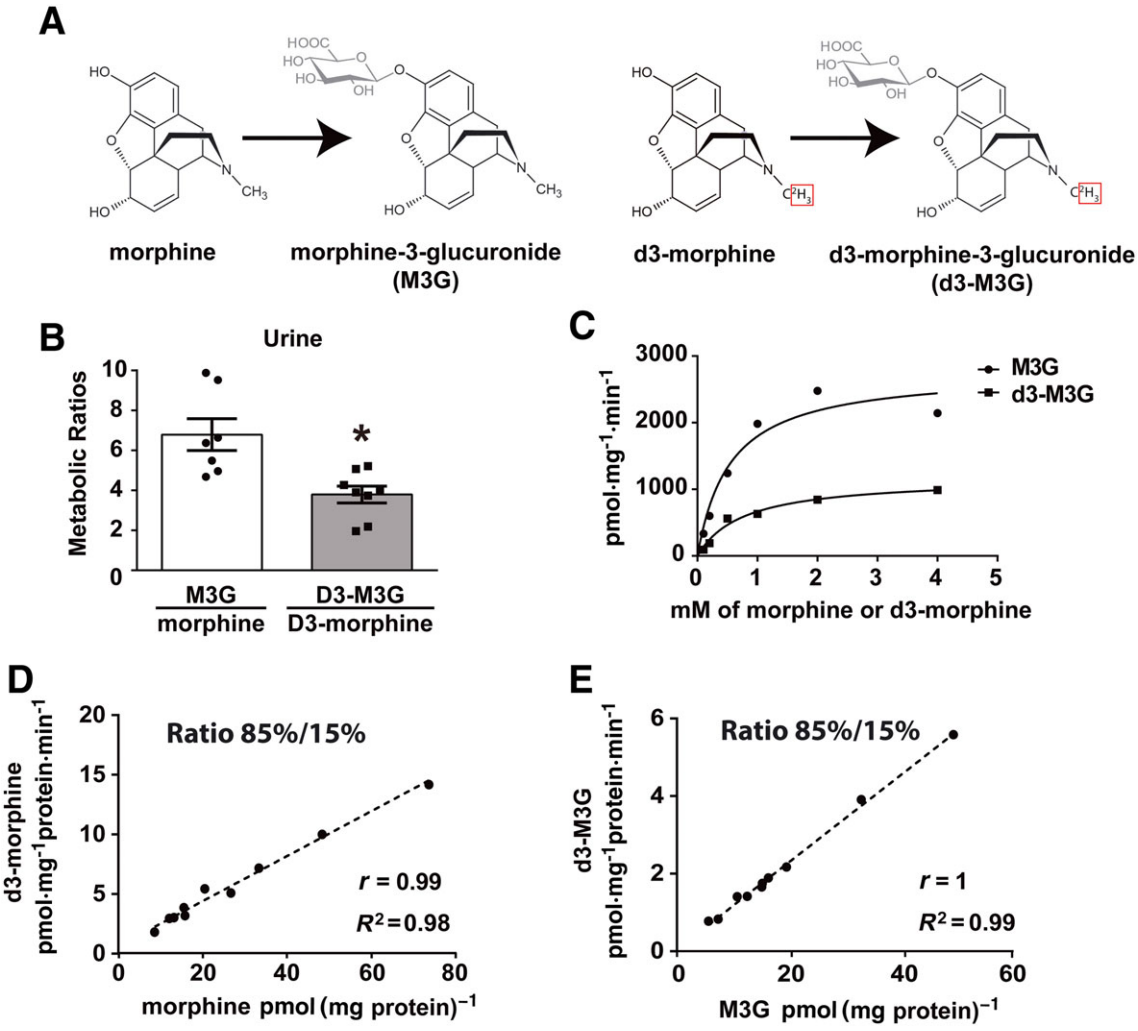

Figure 1

Method validation. (A) Structures of morphine, M3G and their respective d3-labelled counterparts. (B) Metabolic ratios for M3G/morphine and d3-M3G/d3-morphine in the urine of mice having a single injection of morphine or d3-morphine (7.5 mg·kg^{-1}, i.p.). Data expressed as mean ± SEM; n = 10 per group. *$P < 0.05$, significantly different from morphine ratio; Mann–Whitney U-test. (C) Michaelis–Menten kinetics of M3G and d3-M3G formation from morphine and d3-morphine respectively using liver extracts of control mice. Correlation between (D) d3-morphine and morphine, and between (E) d3-M3G and M3G levels in the liver of control mice after injection of a mix of morphine/d3-morphine (85%/15%, w/w, 10 mg·kg^{-1}, i.p.). For both correlations, $P < 0.05$.

baseline response latency was obtained for each mouse. Data are expressed as % maximal possible effect (% MPE) according to the following formula:

$$\%MPE = \frac{(test\ latency) - (baseline\ \ latency)}{(cut - off\ latency) - (baseline\ latency)} \times 100.$$

Tissues, plasma and urine recovery

Mice were anaesthetized with ketamine/xylazine (ketamine: 17 mg·mL^{-1}, i.p., xylazine: 2.5 mg·mL^{-1}, i.p., 4 mL·kg^{-1}; Centravet, Taden, France). Adequate anaesthesia was ensured by pinching the hind paws with tweezers and observing no motor response. Blood was obtained by the intracardiac route using heparinized syringes (0.5 mL; 22Gx1½ needle). Plasma was prepared from blood recovered in lithium-heparin tubes (BD, ref 367526) by centrifugation at 1300 *g* for 15 min. After decapitation, intrabladder puncture was used to recover urine (22Gx1½ needle) and stored into low-binding microtubes (Sorenson, SafeSeal, ref 27210). Brain and liver were immediately collected.

Cell culture and treatment

The murine microglial cell line BV-2 was a kind gift from Dr Nancy Grant (CNRS UPR3212, Strasbourg, France). BV-2 cells were maintained in RPMI 1640 medium (Thermo Fisher Scientific, Illkirch Graffenstaden, France) supplemented with 10% heat-inactivated FBS (Thermo Fisher Scientific) and antibiotics (penicillin 100 U·mL^{-1}, streptomycin 100 µg·mL^{-1}; Thermo Fisher Scientific) at 37°C under a humidified atmosphere with 5% CO_2.

BV-2 cells (10^6) were seeded in 6 cm diameter culture dish for 24 h in presence of FBS. Then, the medium was replaced with 3 mL of fresh medium containing 20 µM of morphine in the absence of FBS. Conditioned medium was recovered after 48 h for extraction and LC–MS/MS analysis.

Preparation of tissues and fluids

Brain and liver were homogenized with an Ultra Turrax instrument (Ika, Staufen, Germany) in 1 and 5 mL of H_2O, respectively, containing protease inhibitors (cOmplete Mini, EDTA-free, Roche, Basel, Switzerland). The homogenates were then sonicated (2 times 10 s, 90 W) with a Vibra Cell apparatus (Sonics, Newtown, USA) and centrifuged (14 000 *g*, 30 min) at 4°C. Supernatant was recovered and protein concentration determined using the Bradford method (Protein Assay, Bio-Rad, Marnes-la-Coquette, France). In order to quantify opiates, 100 µL of brain or liver extract, plasma or BV2-conditioned media were acidified with 700 µL of 0.5% formic acid (v/v). After centrifugation (14 000 *g*, 15 min, 4°C), supernatants were collected prior to solid phase extraction (SPE). The SPE procedure was performed with a positive pressure manifold (Thermo Electron, Courtaboeuf, France). HyperSep PGC SPE-cartridges (1 cc, 25 mg, Thermo Electron) were first activated with 1 mL of acetonitrile (ACN) and then washed twice with 1 mL of H_2O/formic acid 0.1% (v/v), and samples were loaded on SPE-cartridges. Cartridges were dried for 1 min under vacuum and were washed with 1 mL of H_2O/formic acid 0.1% (v/v). Pre-elution was performed with 1 mL of ACN 2%/H_2O 97.9%/formic acid 0.1% (v/v/v). Elution was performed with 800 µL of ACN 20%/H_2O 79.9%/formic acid 0.1% (v/v/v). Eluates were collected in low binding 1.5 mL tubes and centrifuged (14 000 *g*, 10 min, 4°C). Supernatants were dried under vacuum and resuspended in 100 µL of H_2O/formic acid 0.1% (v/v) prior to MS analysis (see below). Urine (10 µL) was diluted 100-fold with H_2O/formic acid 0.1% (v/v), prior to direct LC–MS/MS analysis. For brain, liver, plasma and BV-2 samples a volume of 10 µL was injected on the HPLC column; for urine, 5 µL of the diluted samples were injected.

Enzymatic activity assay

A total of 250 µg of liver extracts were used to perform morphine glucuronidation enzymatic assays. First, extracts were incubated for 30 min at 4°C in the presence of alamethicin (50 µg·mg^{-1} of protein; Sigma Aldrich, Saint Quentin Fallavier, France) adjusted to a final volume of 112 µL with H_2O. Then, the Enzymatic reaction was performed in 100 mM phosphate buffer (pH 7.4), 4 mM $MgCl_2$, and increasing concentrations of morphine or d3-morphine (0.001, 0.005, 0.01, 0.05, 0.1, 0.2, 0.5, 1, 2, 4 and 6 mM) in a final volume of 200 µL at 37°C for 32 min. After 5 min of equilibration at 37°C, the reaction was started by the addition of UDP glucuronic acid (UDPGA; final concentration 5 mM). Reactions were terminated by precipitation with perchloric acid (0.7% final concentration, v/v). Samples were centrifuged (20 000 *g*, 15 min, 4°C). Supernatants were collected, and 10 µL of supernatant were directly analysed by LC–MS/MS.

K_M and Vmax were obtained from a Michaelis–Menten representation after a non-linear curve fit with the least-squares method using Graphpad Prism 6 software.

LC–MS/MS instrumentation and analytical conditions

Analyses were performed on a Dionex Ultimate 3000 HPLC system (Thermo Scientific, San Jose, USA) coupled with a triple quadrupole Endura mass spectrometer (Thermo Scientific). The system was controlled by Xcalibur v. 2.0 software (Thermo Electron). Samples were loaded onto an Accucore C18 RP-MS column (ref 17626-102130; 100 × 2.1 mm 2.6 µm, Thermo Scientific) heated at 40°C. The presence of morphine, d3-morphine and corresponding 3-O-glucuronides was studied using the multiple reaction monitoring mode (MRM). Elution was performed at a flow rate of 400 µL·min^{-1} by applying a linear gradient of mobile phases A/B. Mobile phase A corresponded to ACN 1%/H_2O 98.9%/formic acid 0.1% (v/v/v), whereas mobile phase B was ACN 99.9%/formic acid 0.1% (v/v). The gradient used is detailed in Table 1.

Electrospray ionization was achieved in the positive mode with the spray voltage set at 3750 V. Nitrogen was used as the nebulizer gas, and the ionization source was heated to 250°C. Desolvation (nitrogen) sheath gas was set to 45 Arb, and Aux gas was set to 15 Arb. The Ion transfer tube was heated at 350°C. Q1 and Q2 resolutions were set at 0.7 FWHM, whereas collision gas (argon) was set to 2 mTorr. Identification of the compounds was based on precursor ion, selective fragment ions and retention times obtained for morphine, M3G, d3-morphine (Alsachim) and d3-M3G standards (Lipomed, Arlesheim, Swiss). Selection of the monitored transitions and optimization of collision energy and RF Lens parameters

Table 1

LC and MS conditions for the purification and the detection of morphine, M3G and their respective d3-labelled counterparts

HPLC gradient							
Time (min)	0	2.5	4.5	6.5	7.5	8	12
% B mobile phase	1	1	30	99	99	1	1

MS ionization, selection, fragmentation and identification parameters						
Compound	**Polarity**	**Precursor (m/z)**	**Product (m/z)**	**Ion product type**	**Collision Energy (V)**	**RF Lens (V)**
Morphine	Positive	285.98	201.11	Quantification	26.23	183
Morphine	Positive	285.98	165.36	Qualification	40.89	183
Morphine	Positive	285.98	181.06	Qualification	36.24	183
d3-morphine	Positive	288.98	201.06	Quantification	26.48	178
d3-morphine	Positive	288.98	153.13	Qualification	43.16	178
d3-morphine	Positive	288.98	165.04	Qualification	39.02	178
M3G	Positive	462.19	286.11	Quantification	30.02	276
d3-M3G	Positive	465.19	289.17	Quantification	29.92	242

Mobile phase A corresponded to ACN 1%/H_2O 98.9%/formic acid 0.1% (v/v/v), whereas mobile phase B was ACN 99.9%/formic acid 0.1% (v/v).

were manually determined (see Table 1 for details). Qualification and quantification were performed in MRM mode. Quantification was obtained using Quan Browser software (Thermo Scientific). For tissues and fluids, alkaloids were quantified using calibration curves of external standards (morphine, M3G, d3-morphine and d3-M3G; 1 fmol to 100 pmol per injection) added to urine, plasma, brain and liver extract of naive mice and submitted to the same procedure described for respective fluids and tissue recovery. Limits of detection (LOD) for morphine, d3-morphine, M3G and d3-M3G were typically around 1–50 fmol, depending on the nature of the matrix (Table S1). LOD was defined as the lowest detectable amount of analyte with a signal-to-noise (S/N) ratio > 3. Limit of quantification was defined as the lowest detectable amount of analyte with a signal-to-noise (S/N) ratio- > 10 (Table S1). All amounts of opiates measured in samples fit within the standard curve limits, with typical analytical ranges (the range of amounts that can be accurately quantified) from 1 fmol–100 pmol to 150 fmol–100 pmol. Recoveries (extraction efficiency) for morphine, d3-morphine, M3G and d3-M3G were respectively 30 ± 7%, 31 ± 8%, 93 ± 5% and 96 ± 5%. Accuracy values (defined as the measured amount of analyte vs. the theoretical added amount in spiked naive samples) for morphine, d3-morphine, M3G and d3-M3G were respectively 118 ± 14%, 119 ± 15%, 93 ± 5% and 96 ± 5%. Precision (CV% between repeated injections of the same sample) values were <1% for same-day measurements and <5% for inter-day measurements.

Data and statistical analysis

The data and statistical analysis in this study comply with the recommendations on experimental design and analysis in pharmacology (Curtis *et al.*, 2018). Due to potential intra-group variations, we used 10 animals per group to achieve a statistically relevant analysis. Statistical analysis was performed using Graphpad Prism 6 software. Groups were compared using the Mann–Whitney *U*-test. A P value < 0.05 was considered statistically significant.

Nomenclature of targets and ligands

Key protein targets and ligands in this article are hyperlinked to corresponding entries in http://www.guidetopharmacology.org, the common portal for data from the IUPHAR/BPS Guide to PHARMACOLOGY (Harding *et al.*, 2018), and are permanently archived in the Concise Guide to PHARMACOLOGY 2017/18 (Alexander *et al.*, 2017a,b,c).

Results

Methodology validation

We have compared, *in vitro*, the glucuronidation of native and d3-morphine (Figure 1A; morphine bearing three ^2H, resulting in a mass excess of 3 Da compared to the parent drug) into M3G and d3-M3G respectively. First, we used microglial cells able to convert morphine into M3G (Togna *et al.*, 2013). The murine microglial BV-2 cell line was incubated with morphine or d3-morphine (20 μM, 48 h). LC–MS/MS analysis revealed a significant decrease of d3-M3G formation *in vitro* by 22% compared to M3G (183 ± 10 vs. 236 ± 13 pmol·mg^{-1} protein, $n = 6$, Mann–Whitney *U*-test). A similar result was also observed *in vivo* in mouse urine after acute injection of morphine or d3-morphine. Urine d3-M3G was significantly lower than M3G (400 ± 70 vs. 458 ± 60 nmol·mL^{-1}) while d3-morphine was higher than morphine (114 ± 18 vs. 76 ± 12 nmol·mL^{-1}) despite both groups receiving an equal dose of the parent drug (7.5 mg·kg^{-1}, i.p.; Table S2). Accordingly, the metabolic ratio of d3-M3G/d3-morphine was almost reduced by half compared to that of M3G/morphine (Figure 1B and Table S2, $P < 0.05$, Mann–Whitney *U*-test).

In vitro experiments performed using liver extracts from control mice revealed a V_{max} of 2775 pmol·mg^{-1} protein·min^{-1} and a K_M of 0.54 mM for morphine glucuronidation, while d3-morphine glucuronidation exhibited a V_{max} of 1172 pmol·mg^{-1} protein·min^{-1} and a K_M of

0.76 mM (Figure 1C). Thus, it is likely that d3-M3G formation is decreased *in vitro* and *in vivo* compared to native M3G, at least in part due to altered enzyme kinetics.

As a substitution of deuterium for the N-methyl hydrogens of morphine (Figure 1A) decreases its analgesic effect almost by half (Elison *et al*., 1961), we chose for our following *in vivo* experiments to use a mix of morphine/d3-morphine (85%/15%, w/w, 10 mg·kg^{-1} i.p.) to ensure both an acceptable level of antinociception in mice and reliable quantification of d3-morphine and its metabolite d3-M3G. After i.p. administration of this mixture to mice, good correlations between liver morphine and d3-morphine (Figure 1D) as well as between M3G and d3-M3G were obtained (Figure 1E). This clearly shows that, despite reduced d3-morphine glucuronidation compared to morphine (Figure 1B, see also metabolic ratios in Table 2 and Figure S1A), individual variations in morphine metabolism are accurately reflected by d3-morphine metabolism. Therefore, we conclude that (i) d3-morphine can be used to quantify newly produced d3-M3G and that (ii) a 85%/15% ratio of morphine/d3-morphine can be used to study morphine glucuronidation and CNS uptake alterations *in vivo*.

We then determined if chronic morphine treatment for 8 days altered *in vivo* morphine metabolism (Figure 2A). The hot plate test was used to monitor the onset of morphine analgesic tolerance 30 min after injection. On the ninth day, a mix of morphine and d3-morphine (85%/15% w/w, 10 mg·kg^{-1} i.p.) was injected and animals were killed 90 min later. The mixture containing 15% of d3-morphine was only slightly less effective than morphine alone. Indeed, morphine-naive animals reached 71 ± 10% MPE following injection of this mix on day 9 (Figure 2B) whereas naive mice injected with pure morphine reached 100% of MPE. Regardless of whether they were injected with 100% morphine or a 15%/85% mix of d3-morphine and morphine on day 9, morphine-tolerant animals returned to % MPE values similar to those in naive saline-treated mice.

Morphine glucuronidation and brain uptake in tolerant mice

With this protocol, morphine and M3G amounts found in tolerant mice may include morphine and M3G resulting from previous injections (days 1 to 8) (Rubovitch *et al*., 2009) while d3-morphine and d3-M3G levels reflect only CNS uptake and catabolism due to the last injection (day 9). Therefore, only results for d3-labelled molecules will be discussed in detail. Full quantification data for all four compounds are available in Table 2. As d3-morphine was injected on the last day, it should be noted that quantification of morphine, M3G, d3-morphine and d3-M3G in mice relies on external standard calibration curves, because no internal standards for absolute quantification are available. We were unable to detect any M6G in our experiments, as mice are known to convert morphine into M3G but not M6G (Oguri *et al*., 1990; Kuo *et al*., 1991; Milne *et al*., 1996; Zelcer *et al*., 2005; Xie *et al*., 2017).

In the brain, morphine and M3G contents were similar in chronically treated animals and control mice (Mann–Whitney *U*-test, $P > 0.05$). *De novo* synthesis of d3-M3G, corresponding solely to d3-morphine glucuronidation on day 9, was not modified in tolerant animals, compared to naive mice receiving only morphine/d3-morphine on the last day. Accordingly, the brain metabolic ratio (d3-M3G/d3-morphine)

Table 2

Quantification of morphine, M3G, and respective d3-labelled analogues in the brain, liver, plasma and urine of control and tolerant mice after injection of a mixture of morphine/d3-morphine (85%/15%, w/w, 10 mg·kg^{-1} i.p.) on day 9

	Brain (pmol·mg^{-1} protein)				Metabolic ratio	
	Morphine	d3-morphine	M3G	d3-M3G	M3G/morphine	d3-M3G/d3-morphine
Control	33 ± 5.8	6.3 ± 1.1	3.7 ± 0.6	0.4 ± 0.07	0.13 ± 0.02	0.07 ± 0.01*
Tolerant	22 ± 2.3	4.0 ± 0.4	2.8 ± 0.3	0.3 ± 0.03	0.14 ± 0.01	0.08 ± 0.01*
	Liver (pmol·mg^{-1} protein)				Metabolic ratio	
	Morphine	d3-morphine	M3G	d3-M3G	M3G/morphine	d3-M3G/d3-morphine
Control	27 ± 6.4	5.8 ± 1.2	18 ± 4.1	2.0 ± 0.47	0.82 ± 0.13	0.4 ± 0.07*
Tolerant	27 ± 3.7	5.3 ± 0.7	14 ± 2.3	1.5 ± 0.28	0.59 ± 0.09	0.32 ± 0.05*
	Plasma (pmol·mL^{-1})				Metabolic ratio	
	Morphine	d3-morphine	M3G	d3-M3G	M3G/morphine	d3-M3G/d3-morphine
Control	1254 ± 401	261 ± 80	1892 ± 406	234 ± 56	2.27 ± 0.42	1.25 ± 0.24
Tolerant	679 ± 119	129 ± 19	1352 ± 202	162 ± 24	2.66 ± 0.65	1.46 ± 0.26
	Urine (nmol·mL^{-1})				Metabolic ratio	
	Morphine	d3-morphine	M3G	d3-M3G	M3G/morphine	d3-M3G/d3-morphine
Control	270 ± 72	55 ± 14	954 ± 124	110 ± 14	4.61 ± 0.6	2.53 ± 0.31*
Tolerant	273 ± 72	56 ± 15	1471 ± 395	174 ± 51	5.49 ± 0.51	2.98 ± 0.26*

Data expressed as mean ± SEM, $n = 10$.
*$P < 0.05$, significantly different from M3G/morphine ratio; Mann–Whitney *U*-test.

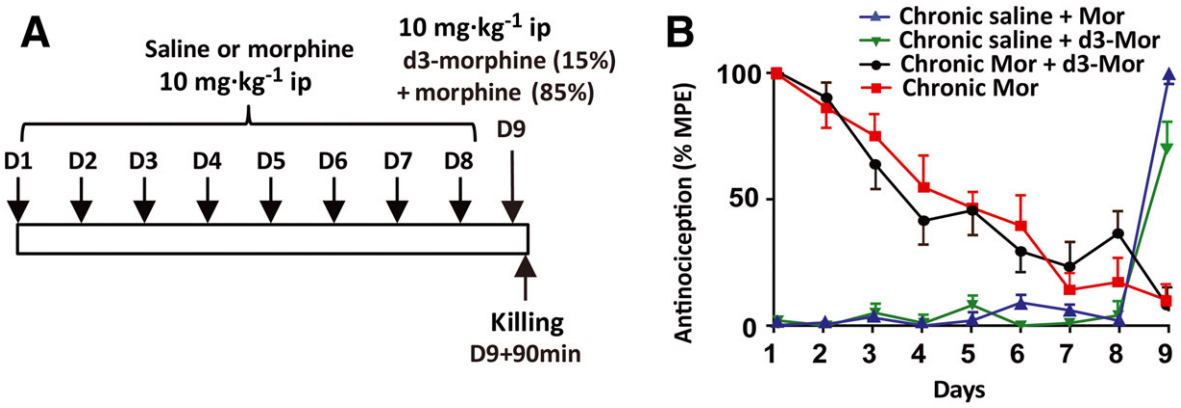

Figure 2

Tolerance induction protocol. (A) Protocol of morphine tolerance induction across days 1 to 8 (D1–D8, 10 mg·kg^{-1} i.p.) and a single injection of 85%/15% morphine/d3-morphine (w/w, 10 mg·kg^{-1} i.p.) on day 9. Two additional groups received only chronic morphine or chronic saline (not shown). (B) Development of morphine tolerance. Antinociception is expressed as % maximum possible effect (% MPE) on the hot plate test observed 30 min after morphine or saline injection across days. Values of MPE are expressed as mean ± SEM; n = 10 mice per group.

was similar in control and tolerant animals (Figure 3A and Table 2). As expected, d3-morphine glucuronidation was reduced in the brain of control and tolerant mice, as reflected by the reduced d3-M3G/d3-morphine ratios compared to M3G/morphine ratios (Figure 3B and Table 2). However, as seen with the liver in our validation study (Figure 1D and 1E), there were strong correlations between brain morphine and d3-morphine levels (Figure 3C) and between M3G and d3-M3G contents in control and tolerant animals (Figure 3D). Together, these results demonstrate that analgesic tolerance was not associated with an up-regulation of morphine glucuronidation in the brain. The presence of similar amounts of d3-morphine in the brain of naive and tolerant mice also shows that morphine BBB permeability is not globally altered.

In order to determine if morphine glucuronidation was modified at the peripheral level, we have analysed liver tissues (Figure 4A), plasma (Figure 4B) and urine (Figure 4C) of control and tolerant mice. Briefly, neither d3-morphine nor d3-M3G levels, nor d3-M3G/d3-morphine metabolic ratios were altered in tolerant animals (Figure 4 and Table 2). Despite reduced glucuronidation of d3-morphine compared to native morphine (Figure S1A–C), LC–MS/MS analysis revealed again remarkable correlations between morphine and d3-morphine levels (Figure S2A–C) and between M3G and d3-M3G contents (Figure S3A–C) in the periphery. Together, these results demonstrate that peripheral morphine glucuronidation was not affected in tolerant mice.

Discussion

Effect of morphine N-methyl deuteration on glucuronidation activity

Different studies have described important differences in the metabolism of various stable isotope-labelled drugs *in vitro* and *in vivo* (Mutlib, 2008; Sanderson, 2009; Schellekens *et al.*, 2011). Replacement of hydrogen with deuterium may lead to significant alterations of drug metabolism and cause changes in the biological effects of drugs, including altered metabolism, pharmacokinetics and toxicity profiles (Timmins, 2014). Such changes are called kinetic isotope effects (see Atkins and de Paula, 2006; Guengerich, 2017). In the case of d3-morphine, a reduction in the rate of oxidative N-demethylation and a weakening of the binding to the enzyme active centre have been described *in vitro* (Elison *et al.*, 1961). However, no data are currently available on the effects of deuteration on drug glucuronidation by UGT enzymes. Because of the lack of a crystal structure of the UGT N-terminal domain, which is the region involved in substrate binding, our current understanding of UGT-morphine interactions and activity remains limited. The three deuterium atoms located on the N17-methyl group of morphine were not expected to influence the glucuronidation step involving the C3-OH because these two groups are at opposite ends of the morphine skeleton (Figure 1A). Therefore, the effect of N-methyl deuteration on glucuronidation is a secondary kinetic isotope effect (SKIE), because no bond to the deuterium substituted atom is broken or involved in a modification (Atkins and de Paula, 2006). SKIEs are usually much smaller than primary kinetic isotope effects and are largely determined by the vibrations of the carbon-deuterium bond (Westaway, 2006). In our case, the SKIE due to deuteration of the N17-methyl group revealed a major involvement of that moiety in UGT catalytic activity. While it may seem counterintuitive at first, a study of UGT2B7-morphine interactions (Coffman *et al.*, 2003) provides plausible structural arguments to explain this phenomenon. Indeed, binding of morphine to the N-terminus of UGT2B7 involves a pocket made of amino acids 96 to 101. Amino acid 99 (Asp) was critical for morphine binding to UGT2B7, which was predicted to occur between Asp^{99} and the morphine nitrogen. Additional structural studies (i.e. crystallography) are required to fully understand how the morphine N17 interacts with the Asp^{99} of the N-terminal domain of the UGT. However, since the crucial amino acid for morphine binding interacts

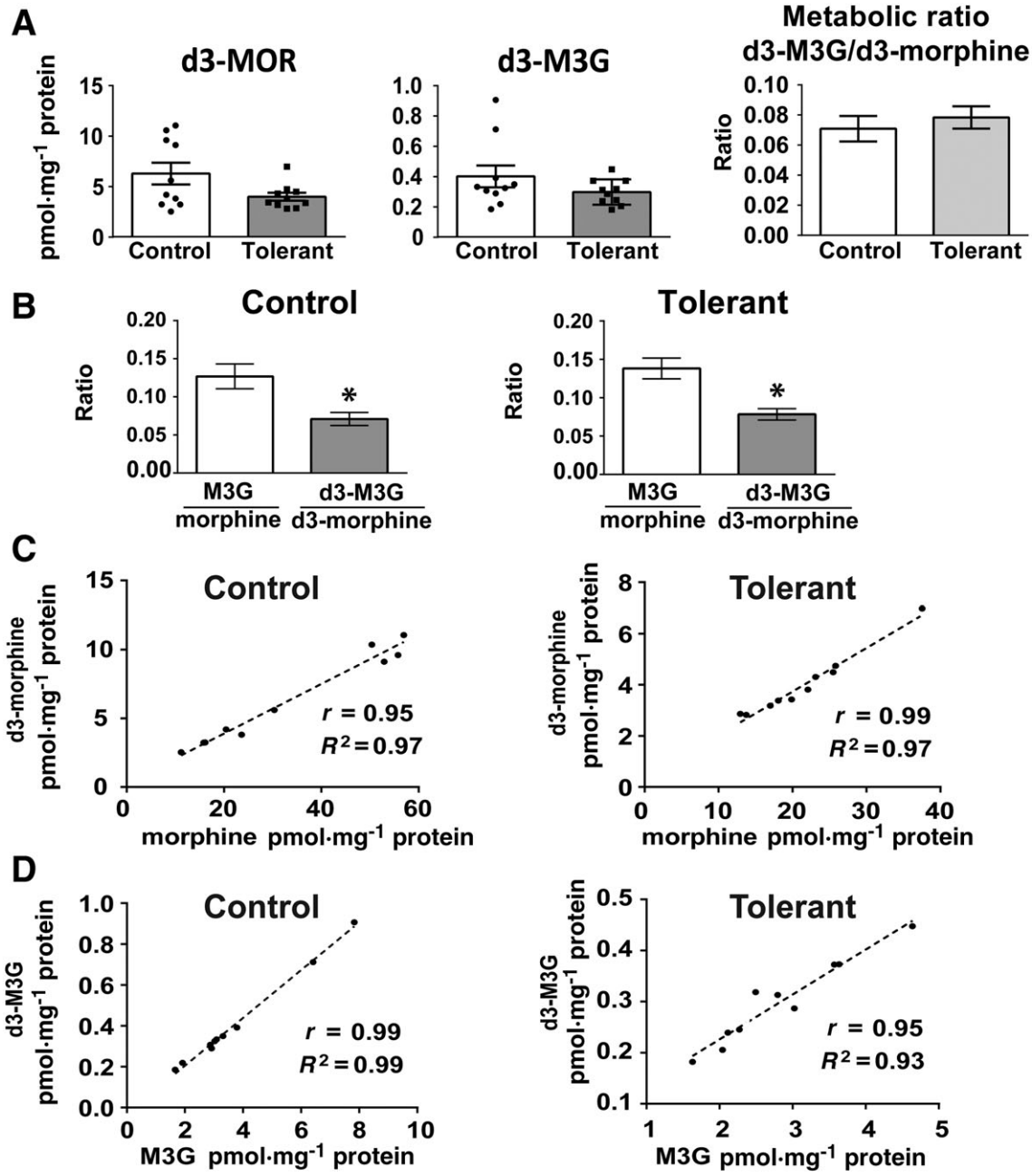

Figure 3

Morphine brain uptake and glucuronidation is not altered in tolerant mice. Quantification was done in control and morphine-tolerant mice 90 min after a single injection of a mix of morphine/d3-morphine (85%/15%, w/w, 10 mg.kg^{-1} i.p.) on day 9. (A) LC–MS/MS quantification of brain d3-morphine and d3-M3G and corresponding metabolic ratios in control and tolerant animals. (B) Brain M3G/morphine and d3-M3G/d3-morphine ratios of control and tolerant mice. Each data point represents one animal and data are expressed as mean ± SEM; $n = 10$. *$P < 0.05$, significantly different from morphine ratio; Mann–Whitney *U*-test. (C) Correlation between brain amounts of d3-morphine and morphine. (D) Correlation between brain amounts of d3-M3G and M3G. Spearman's *r* and R^2 of the linear regression fit are indicated in each panel; $P<0.05$, for each correlation.

with the nitrogen, it seems plausible that alteration of the N-methyl group (e.g. triple deuteration) would affect this binding and therefore glucuronidation activity. Furthermore, the fact that d3-morphine is surprisingly much less analgesic than native morphine (Elison *et al.*, 1961) suggests that N-methyl deuteration affects morphine pharmacology to a stronger extent than that usually seen with deuterated drugs. Overall, this shows the importance of assessing the effects of stable isotope labelling on drug pharmacology in metabolism studies using such drugs.

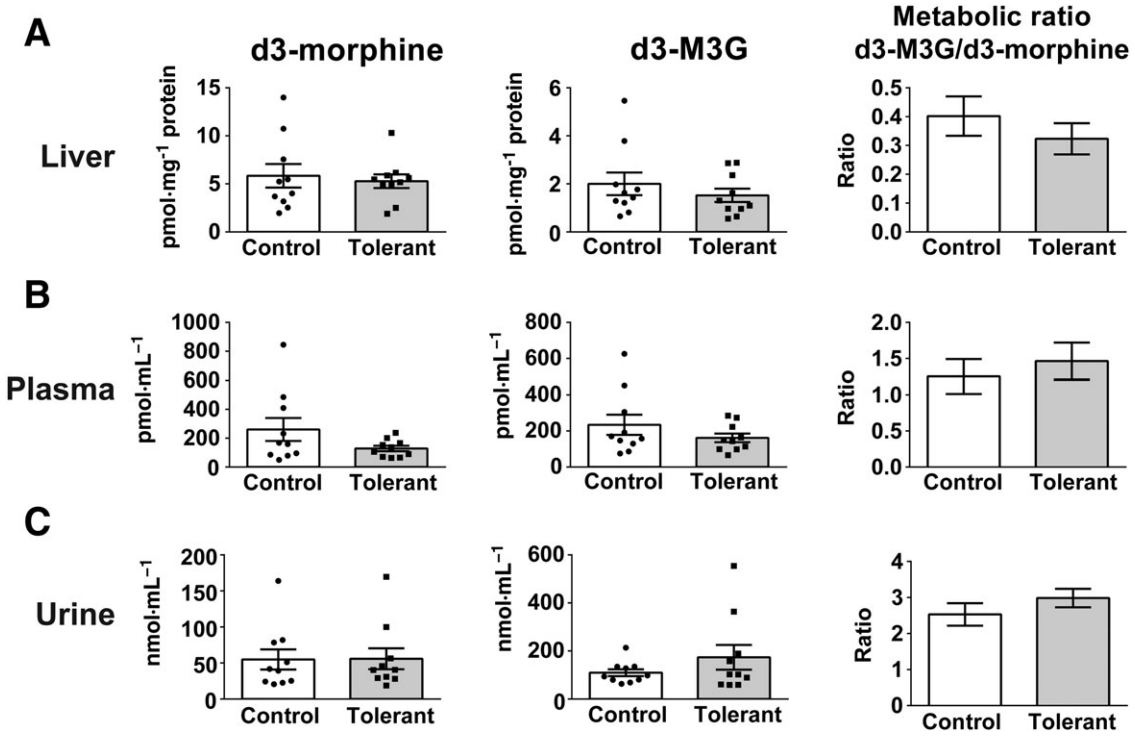

Figure 4

Peripheral morphine glucuronidation is not altered in tolerant mice. LC–MS/MS quantification of d3-morphine and d3-M3G was done in control and morphine-tolerant mice 90 min after a single injection of a mix of morphine/d3-morphine (85%/15%, w/w, 10 mg·kg^{-1} i.p.) on day 9. (A) Liver. (B) Plasma. (C) Urine. Metabolic ratios correspond to d3-M3G/d3-morphine. Each data point represents one animal, data expressed as mean ± SEM; n = 10 for all samples; Mann–Whitney U-test.

Deuterated drugs as probes for metabolic studies

We have used an *in vivo* methodological approach enabling to monitor CNS stable isotope-labelled drug uptake and degradation during chronic treatment without interference from ongoing drug administration and metabolite presence due to previous injections. Our protocol was adapted from earlier pharmacological studies using stable isotopes (Malik *et al.*, 2003).

The use of a deuterated analogue for metabolic studies calls for controls in order to determine if the stable-isotope labelled drug behaves in the same way as the reference drug. Notably, it is important to determine: (i) the distribution of the precursor drug and its metabolites *in situ*; (ii) whether the native and stable isotope-labelled drugs undergo similar metabolism *in vivo*; (iii) kinetic parameters (K_M, Vmax) for normal and stable-isotope labelled drugs *in vitro*. If the stable isotope-labelled drug exhibits altered pharmacokinetics, it is essential to determine if this will hinder its use as a probe for the native drug's metabolism. The key issue is whether an injection of stable isotope-labelled drug can mimic individual variations in the native drug's metabolism and disposition *in vivo*. In other words, poor and extensive metabolizers of the native drug should also be poor and extensive metabolizers of the stable isotope-labelled drug.

In the present study, d3-morphine metabolism was altered compared to morphine, but we conclude that it was without any interference with our study. Indeed, after i.p. administration of a mixture of morphine and d3-morphine to mice, remarkable correlations between the levels of morphine and d3-morphine as well as between M3G and d3-M3G were observed in all tissues and fluids (Figures 1C, 1D, 3C, 3D, S2 and S3). Our results show that intrinsic differences in metabolism and physiological properties of stable-isotope labelled drugs compared to the native drugs do not preclude their use as metabolic probes.

LC–MS/MS can identify and quantify low amounts of target compounds with a selectivity of >99% (Manes *et al.*, 2015). It allows the analysis of metabolites of interest following acute or chronic treatments and can be easily applied to study the pharmacokinetics of other homeostatic and metabolic processes by adding heavier precursors (^{2}H, ^{13}C …) at defined times. One limitation of our study is the fact that we assessed morphine metabolism at a single time point and in the whole brain. However, *in vivo* longitudinal studies in animals and humans could be achieved using different stable isotope-labelled precursors (d3-morphine, d6-morphine …) and periodical MS analysis of blood, urine, as well as microdialysis samples. Furthermore, while our protocol for the induction of tolerance is widely accepted (Ueda *et al.*, 1997; Elhabazi *et al.*, 2014), it does not

accurately reflect the morphine treatment schedule in patients. Thus, our study's clinical relevance could benefit from using extended release morphine formulations or from increasing the number of injections per day. In the latter case, we observed identical results when injecting mice twice per day with 10 $mg{\cdot}kg^{-1}$ (data not shown).

In addition to monitoring drug metabolism, our methodology can assess modifications of BBB permeability after chronic treatment. No differences of d3-morphine brain content were observed between acute and chronic morphine treatments. However, the use of stable isotope-labelled drugs needs to be carefully performed because BBB-permeability for the deuterated analogue might be altered. For instance, Dewar and colleagues have shown an increased penetration of deuterated β-phenylethylhydrazine into the rat brain compared to non-deuterated β-phenylethylhydrazine (Dewar *et al.*, 1988; Timmins, 2014). This might be explained by a differential affinity between deuterated and non-deuterated compounds for specific BBB transporters.

Notably, our approach could be applied to therapeutic drug monitoring (TDM) and enzyme autoinduction assays during chronic treatments (Sinz *et al.*, 2008; Kang and Lee, 2009). Indeed, as stable isotope-labelled drugs are analytically distinct but pharmacologically similar to the parent therapeutic molecule, they allow more accurate measurements without disturbing ongoing treatment. While stable isotope-labelled drugs are currently too costly for routine use in the clinic, they could be useful as probes for *in vivo* metabolic and BBB permeability alterations in the case of therapeutic drugs that do not respond well to classical TDM (e.g. drugs with extensive half-lives).

M3G involvement in chronic morphine side effects

Kinetic parameters obtained on liver extracts (K_M of 0.54 mM and Vmax of 2.77 $nmol{\cdot}mg^{-1}$ $protein{\cdot}min^{-1}$, Figure 1C) are in agreement with published values (K_M of 0.42 mM and Vmax of 19 $nmol{\cdot}mg^{-1}$ $protein{\cdot}min^{-1}$; Shiratani *et al.*, 2008). The lower Vmax might be due to the fact that we used liver extracts instead of purified liver microsomes, which are enriched with UGT enzymes. Similarly, our data are consistent with previous studies reporting morphine and M3G plasma levels (low μM ranges) 90 min after morphine injection (Zelcer *et al.*, 2005; Andersen *et al.*, 2009).

Neither morphine CNS uptake nor its glucuronidation was altered in chronically treated animals, compared to acutely treated mice. This result argues against a role of global M3G overproduction in the development of tolerance and hyperalgesia following morphine chronic treatment. However, since we used whole brain samples, we cannot rule out local CNS alterations in morphine metabolism and uptake. Furthermore, M3G could still play a role in morphine analgesic tolerance and hyperalgesia through alterations of its effects at the receptor level [i.e. through **toll-like receptor 4 (TLR4)** and μ receptors (Lewis *et al.*, 2010; Roeckel *et al.*, 2017)]. Therefore, additional studies focusing on local (e.g. in the periaqueductal gray, the main site of morphine analgesia) M3G synthesis and modulation of TLR4/μ receptor signalling are needed to clarify whether or not M3G is involved in morphine tolerance and hyperalgesia.

Previous studies of a potential alteration in BBB function following morphine treatment used either *in vitro* (Strazza *et al.*, 2016) or *in vivo* approaches (blue Evans-albumin tracer, [^{131}I]-albumin and [^{14}C]-sucrose) (Sharma and Ali, 2006; Yousif *et al.*, 2008; Chaves *et al.*, 2017). Contrasting effects have been described (i.e. increase or no increase of BBB permeability). Our *in vivo* approach, which we believe is more physiological than previous studies, suggests that global morphine BBB permeability is not altered and does not play a role in the development of negative side effects following chronic morphine.

In conclusion, we have confirmed the importance of deuterated compounds as a means of studying metabolic adaptations that follow chronic drug administration. Interestingly, N-methyl deuteration affected morphine pharmacology to a greater extent than that usually seen with deuterated drugs. This highlights the importance of the proper characterization of kinetic isotope effects, when using stable isotope-labelled drugs for pharmacological studies. To our knowledge, no study before ours had directly investigated morphine uptake and glucuronidation in the CNS of tolerant animals. Using d3-morphine in the mouse, we have shown that analgesic tolerance is not linked to an increase in morphine glucuronidation into M3G or to a modification of the drug's global blood–brain barrier permeability.

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Author contributions

Conceptualization was performed by Y.G., A.L., I.W., M.O.P., P.P., P.D., A.C., M.L., A.V.D. and S.C. Y.G., A.L., I.W., J.M., M.O.P., A.V.D., F.G. and S.C. presented the methodology. A.L., T.M., I.W., J.M., V. C, F.G., F.D. and Y.G. performed the investigation. Writing – original draft was written by Y.G., I.W., M.O.P. and A.C. Writing – review and editing were done by P.P., P.D., M.L., T.M., A.V.D. and S.C. Y.G. and M.O.P. presented the funding acquisition. Resources were supplied by Y.G., M.L., A.V.D., S.C. and P.P. Supervision was performed by Y.G.

Conflict of interest

The authors declare thave no conflicts of interest.

Declaration of transparency and scientific rigour

This Declaration acknowledges that this paper adheres to the principles for transparent reporting and scientific rigour of preclinical research recommended by funding agencies, publishers and other organisations engaged with supporting research.

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Supporting Information

Additional supporting information may be found online in the Supporting Information section at the end of the article.

https://doi.org/10.1111/bph.14454

Figure S1 Metabolic ratios for M3G/morphine and d3-M3G/d3-morphine in control and tolerant mice. Quantifications were done in control and morphine-tolerant mice treated with a single injection of a mix of morphine/d3-morphine (85%/15%, m/m) on day 9. (A) Liver. (B) Plasma. (C) Urine. Data expressed as mean ± SEM; n = 10 for all samples; Mann–Whitney. U test. *$P < 0.05$.

Figure S2 Correlation between amounts of d3-morphine and morphine in tissues and fluids of control and tolerant mice. (A) Liver. (B) Plasma. (C) Urine. Spearman's r, P-value and R^2 of the linear regression fit are indicated in each panel; n = 10 for all tissues.

Figure S3 Correlation between amounts of d3-M3G and M3G in tissues and fluids of control and tolerant mice. (A) Liver. (B) Plasma. (C) Urine. Spearman's r, p-value and R^2 of the linear regression fit are indicated in each panel; n = 10 for all tissues.

Table S1 Limits of detection (LOD), limits of quantification (LOQ) and reportable ranges for morphine, d3-morphine, M3G and d3-M3G in brain, liver, plasma and urine samples. LOD was defined as the lowest detectable

amount of analyte with a signal-to-noise (S/N) ratio > 3. LOQ was defined as the lowest detectable amount of analyte with a signal-to-noise (S/N) ratio > 10. Data are presented as the mean ± SEM of 5 measurements. Reportable range reflects the range of analyte amounts that fit within the linear standard curve limits.

Table S2 Quantification of morphine, M3G, and respective d3-labelled analogs in mouse urine after acute injection of morphine or d3-morphine (7.5 mg/kg, i.p.). Data expressed as mean ± SEM. Mann–Whitney's U-test; **P*-value < 0.05 for the comparison of metabolic ratios.